Increase in Activated Macrophages in Balb/c Mice As a Result of Leishmanial Infection

An Honors Thesis (ID 499)

by

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Abstract

The effects of leishmanial infection on various parameters of a susceptible strain of mice, Balb/c, were studied. Specifically, we were interested in the change in percent of Ia positive peritoneal macrophages as the disease progressed. Typically, in healthy animals, peritoneal macrophages express low percentages of Ia. Previous studies have shown, however, that in an autoimmune disease, such as found in MRL-lpr mice and infections such as Listeria monocytogenes, there is a dramatic increase in Ia expression on macrophages. In our work, Balb/c mice were injected in the footpad with an inoculum of Leishmania major and the Ia expression on peritoneal macrophages was followed, along with several other disease parameters, throughout the course of the infection. These parameters included foot pad size, spleen weight, isolation of L. major from spleen culture, and regional lymph node weight. Results of this investigation indicate that expression of Ia does greatly increase over the course of the infection and occurs prior to splenomegaly and significant lymphoproliferation, which are characteristic manifestations of the infection. This suggests that the Ia expression may have some, as yet unknown, influence on immune regulation and the failure of this mouse strain to develop a protective immunity to the parasite.

Introduction

Macrophages are known to present antigen to the T cells in conjunction with Ia molecules found on the cell surface (1). Resident murine peritoneal macrophages from normal animals have been shown to have very low percentages of Ia positive cells. Beller, et. al. (2) reports that between 5-30% of peritoneal exudate macrophages are Ia positive. Confirming this, Schwartz, et. al. (3) report that 8-15% of these cells are Ia positive. During certain autoimmune, lymphoproliferative diseases and chronic infections with intracellular parasites, the percent of Ia expression has been shown to increase dramatically (2). Beller et. al. reported that the initial percent of Ia positive peritoneal macrophages before infection with Listeria monocytogenes was 7.1%. After infection, the percent increased to between 20-86%. This increase may be due to either an increased need for antigenic presentation (4) or from stimulation by a soluble product of activated T cells (such as γ-IFN) (1,4,5). It has also been shown that in the case of an autoimmune, lymphoproliferative disease in aging MRL-lpr mice, the Ia positive peritoneal macrophages increased to an average value of 36% after onset of the disease. This increase is significant when compared to the initial percentage of approximately 9% in young, still healthy mice (6). We wished to determine the rate of increase of the Ia positive macrophages in the case of Leishmania major infection of Balb/c mice. These mice are known to be highly susceptible to this parasite and, following infection, develop a chronic disease that leads to visceralization, lymphoproliferation and some autoimmune features. This infection is ultimately fatal (7).

It may be that an altered level of Ia expression among peritoneal macrophages is associated with either the development or expression of serious disease in these mice. It is the purpose of this study to determine the possible role of Ia expression on the progression of a leishmanial infection in Balb/c mice over a two month period.

Materials and Methods

Mice. Twenty-nine male Balb/c mice (haplotype Ia^d) were used. (All were bred in our own breeding facilities). The mice were all 8-10 weeks old at the onset of the experiment.

Infection with Leishmania. The Leishmania major used were isolated from the spleen of an infected mouse and grown in Medium 199 (Sigma Co.) with 20% fetal calf serum (FCS). The culture had been passed fewer than ten times prior to use. Twenty-two mice were injected with an inoculum of stationary phase Leishmania major promastigotes sub-cutaneously in the left hind foot pad. This stage has been shown to be the most infective one for Balb/c mice (8). The culture was diluted in saline to a concentration of $2x10^7$/ml and 0.05 ml injected. Controls were left uninfected and were used to establish baseline readings for normal levels of all parameters being followed.

Paraformaldehyde Fixative. 1% paraformaldehyde was used to fix the adherent peritoneal cells after incubation. This was made fresh before use from 4% stock paraformaldehyde solution, diluted in a phosphate (PO_4) buffer solution each trial. The 4% solution was made every two weeks by dissolving 2 grams of paraformaldehyde in 47.5 ml of 0.1 M PO_4 buffer (pH 7.3) solution. This was done by heating the solution to 70 degrees Celsius with continual stirring, then adding 2.5 ml of 0.001 M $CaCl_2$. The solution was cooled and filtered through a Buchner funnel with Whatman #1 filter paper. After filtering, another 5 ml of 0.1 M PO_4 buffer solution was added. This 4% solution was then refrigerated and diluted on the day of the trial with additional PO_4 buffer.

Monoclonal Antibody. Monoclonal anti-Ia^d and anti-Ia^b were graciously provided by Dr. E. Unanue's laboratory and were used in order to detect Ia expression on the macrophages. The anti-Ia^d antibody is specific to the Ia^d haplotype found in Balb/c mice used in our experiments. Previous experiments in our lab had shown that the anti-Ia^d did specifically detect the Ia of Balb/c mice.

Briefly, the specificity of the reaction with anti-Ia and the fluorescein labeled $(Fab')_2$ anti-Ig was determined in the following manner. Resident peritoneal macrophages from C57/black (haplotype Ia^b) and Balb/c mice were stained with both anti-Ia^b and anti-Ia^d. Results of this trial showed that the Ia positive peritoneal macrophages of C57/black mice stained only in the presence of anti-Ia^b while the macrophages of the Balb/c mice stained only when in the presence of anti-Ia^d. The $(Fab')_2$ by itself did not non-specifically stain the cells as only a very small percentage of the macrophages present fluoresced when treated with the fluorescent-tagged antibody alone.

The anti-Ia^d used for each trial was diluted fresh on the day of each trial. 50 µl anti-Ia^d was diluted in 450 µl of rabbit serum diluent to a concentration of 100 µg/ml. The rabbit serum diluent contains 10 mg/ml Bovine Serum Albumin and 10% normal rabbit serum in RPMI 1640 medium (Sigma Co.).

$(Fab')_2$ Conjugate. An FITC (Fluorescein Isothiocyanate) conjugated antibody fragment $(Fab')_2$ directed toward the Fc portion of the monoclonal anti-Ia was also provided by Dr. E. Unanue's laboratory. As mentioned above, the $(Fab')_2$ did not stain cells non-specifically. The conjugate was diluted fresh on the day of each trial. 2 µl of $(Fab')_2$ conjugate were diluted in 198 µl of rabbit serum diluent to a concentration of approximately 86 µg/ml.

Procedure for Removal and Staining of Peritoneal Exudate Cells. For each trial, 2 infected mice and 1 normal mouse were sacrificed by asphyxiation with CO_2. The peritoneal exudate cells (PEC's) were collected by lavage using 8-10 ml RPMI 1640 medium supplemented with 5% FCS. The cells were spun in a centrifuge at 2000 rpm for 15 minutes. They were then collected and raised in RPMI 1640 plus 5% FCS, counted, and adjusted to approximately $5x10^6$-$1x10^7$/ml. Coverslips (Bellco. labs) were placed in a 24-well micro-titer plate (Falcon 3047 multiwell tissue culture plate) and 1 ml of the $5x10^6$-$1x10^7$/ml cell suspension was placed in each well (generally 3 wells per mouse were set-up; 2 were stained with anti-Ia^d and the third was used as a control, with PBS in place of the anti-Ia^d). The

micro-titer plate was then spun for 5 minutes in the centrifuge at 1000 rpm to aid in the adherence of the cells to the coverslips. The plate was then placed in an incubator for 2 hours at 37 degrees Celsius and 5% CO_2.

The staining process used was that of Beller, et. al. (2) which was amended slightly. Briefly, the plate was removed from the incubator and each coverslip was washed well with saline. The coverslips were then placed in clean wells with approximately 1 ml of 1% paraformaldehyde for 15 minutes at room temperature. (The 1% paraformaldehyde treatment did not affect detection of Ia and acted to fix the cells on the coverslips) (2). The coverslips were again washed well with saline and then placed cell-side down on a slide containing 15 µl anti-Ia^d or phosphate buffered saline (PBS) as a control. These slides were placed in glass petri dishes (to avoid contamination by the ice) and set on ice for 30 minutes. They were once again washed with saline and placed cell-side down on clean slides containing 15 µl of anti-$(Fab')_2$ conjugated with FITC dye. These were placed in petri dishes and set on ice for 30 minutes. The coverslips were again washed with saline and each was placed cell side up separately in wells of a fresh 24-well micro-titer plate with approximately 1 ml of PBS and refrigerated until the cells could be examined under a Zeiss Fluorescent microscope. At that time, the coverslips were placed cell-side down in approximately 25 µl of PBS in the depression of a hanging-drop slide. Each field of macrophages was counted first under white light and then under UV light. The number of fluorescing macrophages in each field was then counted. Fields were counted until between 200-300 macrophages were studied. The coverslips were then replaced in the micro-titer plate in PBS. This examination was done within 24 hours of completion of the staining procedure.

Spleen Culture. The spleens of the infected mice were removed sterilely after the PEC's were collected. The spleens were weighed and then ground between two glass slides into Medium 199 with 20% FCS. This solution was then placed into culture bottles (Corning 25 cm^2 sterile tissue culture flask) and incubated at 25 degrees Celsius. The

cultures were checked daily for the presence of Leishmania major by microscopic examination of a sample of the culture fluid. Negative cultures were re-incubated for a period of three weeks before being discarded. The number of days between culture and first appearance of Leishmania in the culture fluid was taken as a measure of the level of parasitization of the spleen by the protozoa.

Foot Pads. The injected foot pads of all mice were measured on the day of the trial with a Vernier caliper gauge by measuring the width from top to bottom of the foot pad. This was done to determine rate of swelling of the infected foot and to establish normal levels using the measurements of the control mice.

Lymph Nodes. The left hind leg popliteal lymph nodes of the infected mice were removed and weighed to determine the level of lymphoadenopathy that occurred following infection.

Results

The results of all parameters are reported in Table 1.

Enlargement of Foot Pads. The foot pads of the infected mice greatly enlarged over the course of the experiment (Fig. 1). This particular measurement was used as the primary means to follow the progress of the disease. The foot pads swell throughout the course of the infection and eventually severe lesions and necrosis of the tissues develop. The line graph illustrates the average progressive enlargement of the foot pads in infected animals compared to an average value determined from measurements of normal mice. The points represent the individual foot pad measurement for each infected mouse sacrificed.

Table 2

	Day 6			Day 13			Day 20			Day 27		
	Normal[1]	Sick 1[2]	Sick 2	Normal	Sick 1	Sick 2	Normal	Sick 1	Sick 2	Normal	Sick 1	Sick 2
Spleen Weight	0.10 g	0.14 g	0.12 g	0.10 g	0.11 g	0.11 g	0.10 g	0.08 g	0.11 g	0.09 g	0.10 g	0.12 g
Lymph Node Weight	none seen	0.003 g	0.002 g	none seen	0.018 g	0.011 g	none seen	0.014 g	0.018 g	0.2 mg	0.019 g	0.038 g
Foot Pad Size	0.25 cm	0.25 cm	0.25 cm	0.22 cm	0.30 cm	0.29 cm	0.23 cm	0.35 cm	0.37 cm	0.25 cm	0.40 cm	0.38 cm
Spleen Culture (+/-)	Not Cultured	Never saw Leishmania		Not Cultured	+ Day 15	+ Day 15	Not Cultured	+ Day 4	+ Day 4	Not Cultured	+ Day 4	+ Day 4
Percent Ia Positive Macrophages	6.9%	6.1%	6.1%	7.4%	6.2%	4.4%	6.5%	19.4%	23.8%	14.3% (assay	10% questionable)	17.2%

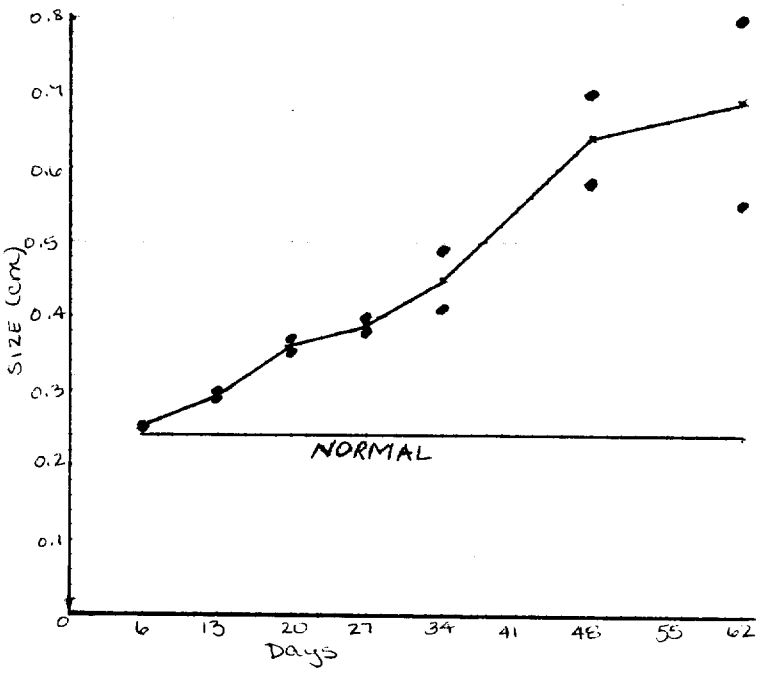
[1]Normal refers to control mice.

[2]Sick refers to injected mice.

	Day 34			Day 48			Day 62		
	Normal	Sick 1	Sick 2	Normal	Sick 1	Sick 2	Normal	Sick 1	Sick 2
Spleen Weight	0.10 g	0.11 g	0.11 g	0.19 g 0.12 g (2nd norm)	0.23 g	0.32 g	0.09 g	0.23 g	0.37 g
Lymph Node Weight	none seen	0.040 g	0.048 g	none seen	0.094 g	0.083 g	none seen	0.090 g	0.17 g
Foot Pad Size	0.25 cm	0.41 cm	0.49 cm	0.25 cm	0.58 cm scabbed sore	0.70 cm scabbed sore	0.22 cm	0.55 cm	0.80 cm
Spleen Culture (+/-)	Not Cultured	+ Day 4	+ Day 4	Not Cultured	+ Day 4	+ Day 2	Not Cultured	+ Day 2	+ Day 2
Percent Ia Positive Macro-phages	7.8%	19.1%	32.2%	19.8%*	34.4%	26.7%	6.5%	32.4%	46%

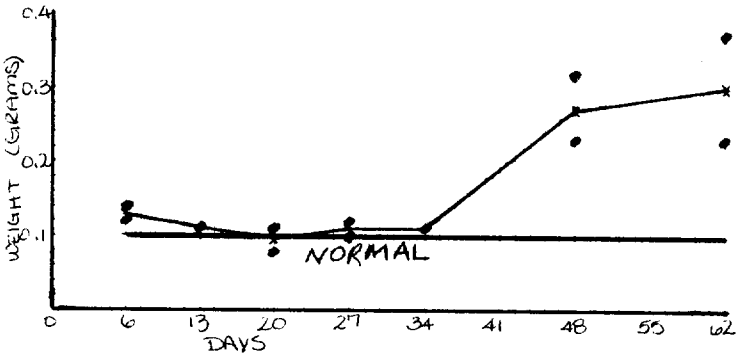
*High level of percent positive macrophages may be due to an infection of the normal mouse as indicated by the enlarged "normal" spleen.

Figure 1



Enlargement of the Spleen. Throughout the course of the experiment, the size and weight of the spleen from the infected animals progressively increased (Fig. 2). The graph demonstrates the increase in weight of the spleens of the infected mice compared to the average values of the weights of the normal mice. After six days, the weights of the infected spleens were 0.12 g and 0.14 g, whereas, the weights of two month infected spleens were 0.23 g and 0.37 g. These measurements represent a two-fold increase in overall size during the course of the experiment (2 months). The line graph shows the average of the two values collected from the infected mice sacrificed on each of the trial dates. The individual points represent the spleen size of each of the infected mice sacrificed on the trial date.

Figure 2



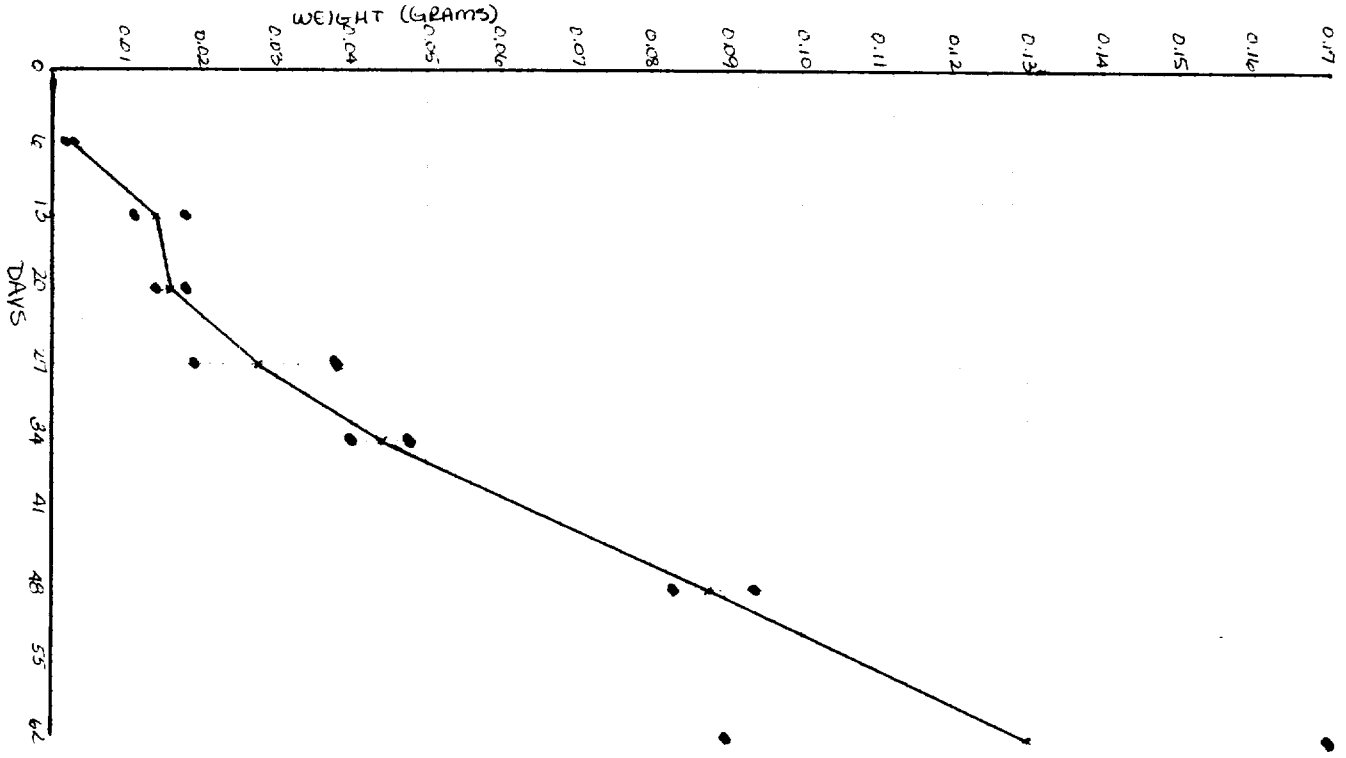
Increased Numbers of Leishmania major. As the infection progressed, the L. major was detected in spleen culture in progressively shorter periods of time (Table 2). Initially, Leishmania were not detected in the infected spleen, but by the conclusion of the experiment, the Leishmania were detected after only two days in culture. The detection period greatly decreased between trial dates 13 and 20 (from 15 days to 4 days), while the spleen weights at the corresponding times did not change dramatically.

Table 2

Trial Day	Day Positive	
6	Not positive	
13	15	15
20	4	4
27	4	4
34	4	4
48	4	2
62	2	2

Enlargement of Lymph Nodes. The lymphatic system is a major contributor to the removal of foreign organisms from the body. The popliteal lymph nodes, which are the primary draining lymph nodes of the foot, demonstrated a dramatic increase in size and weight during the L. major infection (Fig. 3). The control mice had no detectable lymph nodes. Again, the line was plotted by determining the average values for the data collected from each of two mice on the day of the trial.

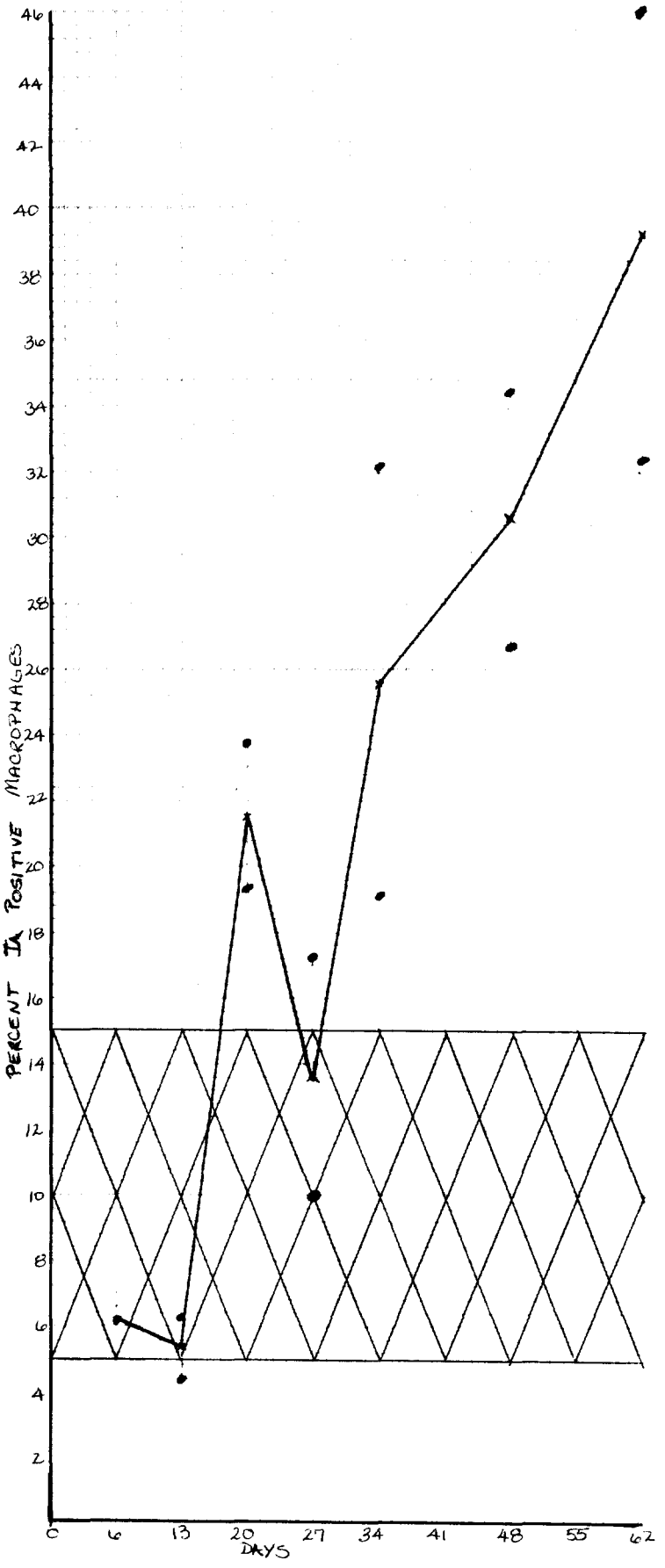
Figure 3



Increase in Percent Ia Positive Macrophages. The amount of Ia present on the macrophages increased progressively and dramatically over the course of the experiment. Initially, only 4-6% of the cells were Ia positive, while at the conclusion of the two month trial period, 32-46% were shown to be positive. This represents an 8-fold increase (Fig. 4).

The values for the seven control mice were averaged. In Fig. 4, the area defined by the cross-hatching indicates the mean of the control values plus two standard deviations. This represents the range of the percent positive Ia of normal mice in our study. The line graph represents the average percent Ia positive macrophage in the infected mice over time. The points represent the percent positive macrophages in the peritoneum in each individual infected mouse examined.

Figure 4


PERCENT IA POSITIVE MACROPHAGES
46
44
42
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
6
13
20
27
34
41
48
55
62
DAYS

Discussion

The results of this investigation demonstrate an increase in Ia positive peritoneal macrophages due to leishmanial infection. This increase was accompanied by enlargement of the popliteal lymph node, spleen, injected foot pad, as well as an ability to isolate Leishmania from the spleen in increasingly shorter periods of time. The enlargement and subsequent necrosis of the foot pad was most likely the result of the proliferation of the injected Leishmania and the consequent immune inflammation at the lesion site. Similarly, the enlargement of the popliteal lymph node and spleen was presumably due to metastasis of the parasite to these locations followed by lymphoproliferation. The increasingly shorter time period required to isolate the Leishmania from the spleen following infection was likely due to an increased number of the parasites in the spleen of animals infected for a longer period of time.

The increase in size of the injected foot pad is used as an indicator of disease progression in infected mice. The increase in size can be positively correlated to the detection of Leishmania in the spleen, increased size of lymph node and a rise in percent Ia positive macrophages. This indicates that these parameters are affected at approximately the same rate during the infection. Table 2 illustrates that the Leishmania metastasized to the spleen in a very short period of time, being detected as early as day 13, and observable in as few as 4 days by trial day 20. This correlates with the rise in percentage Ia positive macrophages, as the first significant increase occurred approximately one week following the first detection of Leishmania in the spleen. Interestingly, the spleen weight, a measure of lymphoproliferation, did not significantly increase until day 48. Also, the lymph node weight only rose slowly until day 27, at which time the weight continued to increase dramatically throughout the conclusion of the experiment. This demonstrates that the increase in activated macrophages occurs prior to marked splenomegaly and lymphoproliferation and quite early in the infection process. Although this finding may not reflect a causal relationship between heightened Ia expression and strain susceptibility, it does indicate an early immune response which is then followed by other signs of a failing immune system.

The focus of this investigation was to determine what effect the infection process had on the Ia presentation of peritoneal macrophages. As shown in this study, this percentage dramatically increased over the course of the disease. Increased Ia expression by macrophages is an indicator of macrophage activation which increases their abilities in many ways, including their ability to interact with T cells and B cells.

One possible explanation which has been offered by several researchers, is that the Ia expression on the macrophages may be influenced by the presence of T cell activated lymphokines, specifically γ-IFN. The macrophages initially stimulate the T cells by presentation of the antigen, and the T cells are thereby stimulated to produce lymphokines which then promote Ia expression of the macrophages. As more T cells are stimulated, more lymphokines are produced, which results in a continual cycle of events (1,4,5).

In general, the increase in percentage of Ia positive macrophages in the L. major infected mice is quite consistent with findings of other researchers working with autoimmune and intracellular parasite models. The percentage of Ia positive macrophages began at a point near that of the normal mice, then increased dramatically as the infection progressed. Initially, the percent was actually below that of the normal mice. This may have been due to the initial processing and internalizing of the parasite by the macrophages. As the parasitic load increased due to proliferation, and a failure of immune mechanisms to check the infection, the percentage also increased.

The early appearance of activated macrophages in the peritoneal cavity may indicate that the macrophages play a large role in the regulation of the immune response. This early appearance may be either related to the cause of the unhindered progression of the infection, or a result of it. An increased number of activated macrophages may cause an improper activation of T cells or an activation of the wrong type of T cells, resulting in a non-protective response. This non-protective response may, in fact, be counter-productive, actually speeding up the progression of the infection. Kurlander and Jones reported that the reduction of Ia positive macrophages in C57Bl/6 mice increased resistance to Listeria monocytogenes (9). This suggests that an increased number of activated macrophages does not always procure protection

against this particular infection, rather aids in its progression. The manner in which this occurs is still not understood.

The increased number of activated macrophages may also be a result of the infection process. This may be due to an activation of the improper type of T cells resulting in the further activation of macrophages. This increase, however, does not necessarily offer protection.

In the case of MRL-lpr mice, the percentage of Ia positive macrophages increased 10-80 fold throughout the development of the autoimmune disease. Lu and Unanue state that this increase is due in part or entirely to the secretion of an Ia inducing factor by activated T cells. This may be either a cause for the development of the autoimmune disease or a result of its progression. Again, the causal relationship has yet to be determined. However, this heightened level of macrophage activation could create a cycle of T cell--macrophage--T cell interactions that could aggravate autoimmune responses and lymphoproliferation (6).

Conclusion and Future Research

In general, the infected mice showed a dramatic increase over time in foot pad size and spleen weight, and Leishmania major was found in the spleens very early in infection. The Ia present on the surfaces of the peritoneal macrophages also increased quite dramatically before evidence of serious disease was manifest. This increase may have been due to presentation of antigen or stimulation of the macrophages by activated T cell products (Y-IFN).

The purpose of our investigation was to determine the correlation of some of the parameters of susceptible mice to the leishmanial disease. In the future, C57/black mice (a strain resistant to L. major infection) will be tested to determine what differences, if any, exist concerning these parameters. These differences may help to explain what processes are involved in the susceptibility of Balb/c mice to the leishmanial infection.

Experiments concerning the effects of prophylactic treatment with Cyclosporine A on the percentage of Ia on macrophages in infected mice are planned in the future, as well. Cyclosporine A is an immunosuppressant drug which has been shown to confer resistance to L. major in Balb/c mice if given prophylactically (7). Results of these experiments may shed some light on

the role that Ia positive macrophages have on the susceptibility of these mice to L. major. Also, testing for the presence of γ-IFN in infected mice may give some information concerning the mechanism involved with this process.

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